The Matrix Metalloproteinase Inhibitor Prinomastat Enhances Photodynamic Therapy Responsiveness in a Mouse Tumor Model

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Abstract

Photodynamic therapy (PDT) clinical results are promising; however, tumor recurrences can occur and, therefore, methods for improving treatment efficacy are needed. PDT elicits direct tumor cell death and microvascular injury as well as expression of angiogenic, inflammatory, and prosurvival molecules. Preclinical studies combining antiangiogenic drugs or cyclooxygenase-2 inhibitors with PDT show improved treatment responsiveness (A. Ferrario et al., Cancer Res 2000;60:4066–69; A. Ferrario et al., Cancer Res 2002;62:3956–61). In the present study, we evaluated the role of Photofrin-mediated PDT in eliciting expression of matrix metalloproteinases (MMPs) and modulators of MMP activity. We also examined the efficacy of a synthetic MMP inhibitor, Prinomastat, to enhance tumoricidal activity after PDT, using a mouse mammary tumor model. Immunoblot analysis of extracts from PDT-treated tumors demonstrated strong expression of MMPs and extracellular MMP inducer along with a concomitant decrease in expression of tissue inhibitor of metalloproteinase-1. Gelatin zymography and enzyme activity assays performed on protein extracts from treated tumors confirmed the induction of both latent and enzymatically active forms of MMP-9. Immunohistochemical analysis indicated that infiltrating inflammatory cells and endothelial cells were primary sources of MMP-9 expression after PDT, whereas negligible expression was observed in tumor cells. Administration of Prinomastat significantly improved PDT-mediated tumor response (P = 0.02) without affecting normal skin photosensitization. Our results indicate that PDT induces MMPs and that the adjunctive use of an MMP inhibitor can improve PDT tumor responsiveness.

Introduction

Photodynamic therapy (PDT) is used clinically for treating a variety of solid malignancies as well as nononcological disorders such as age-related macular degeneration and psoriasis (1, 2). The treatment involves the systemic administration of a photosensitizer followed by irradiation of the targeted lesion with visible light. This multistep procedure initiates the photochemical generation of cytotoxic reactive oxygen species such as singlet oxygen within the treatment field and leads to direct tumor tissue destruction and microvascular disruption (1). The Food and Drug Administration-approved photosensitizer Photofrin (PH), as well as a variety of second generation photosensitizers, is currently used in PDT clinical trials. Therapeutic results are encouraging; however, recurrences can occur and, therefore, methods to improve long-term PDT responsiveness are needed. PDT induces oxidative stress, localized inflammation, and vascular injury within treatment fields, and each of these responses can lead to increased expression of angiogenic factors, cytokines, and survival molecules (1, 3). Overexpression of these molecules can activate pathways associated with tumor recurrence. We recently documented increased expression of vascular endothelial growth factor and prostaglandin E2 in murine tumors treated with PH-mediated PDT and demonstrated that combination procedures using inhibitors of vascular endothelial growth factor or cyclooxygenase-2 improved the therapeutic efficacy of PDT (4, 5).

Growth of solid tumors depends on the formation and development of new blood vessels concomitant with the degradation of the extracellular matrix (6). Information obtained by numerous laboratories directly links the expression of matrix metalloproteinases (MMPs) with these processes (7, 8). MMPs are members of a multigene family of zinc-containing enzymes that function under both physiological and pathological conditions (9). These proteinases share sequence homology and are grouped into different subfamilies according to structure, substrate specificity, and cellular localization (9). Cytokines, growth factors, oncogenes, and reactive oxygen species are among the stimuli that activate MMP transcription (8–10). The translated proteins are usually expressed in an inactive or latent proenzymatic form that requires the proteolytic cleavage of an NH2 terminus peptide domain to be converted to a biologically active proteinase. A positive correlation exists between expression/activation of MMPs and tumor angiogenesis, growth, invasion, and metastatic potential (8, 9). In solid tumors, MMPs are often expressed by stromal cells and macrophages rather than by tumor cells (8, 11). The in vivo activity of MMPs is regulated in part by endogenous tissue inhibitors of MMPs or TIMPs (12). An imbalance in the expression and activation of TIMPs and MMPs leads to modifications in tumor growth.

In the present study, we evaluated expression patterns, biological activity, and cellular sources of MMPs in a murine tumor model after PH-mediated PDT. We also examined the role of MMP activity in modulating tumor responses after PDT using the synthetic MMP inhibitor Prinomastat. Our results indicate that PDT-treated tumors have increased expression of MMPs and that pharmacological inhibition of MMPs using Prinomastat can selectively increase in vivo PDT tumoricidal activity.

Materials and Methods

Drugs. The photosensitizer PH was a gift from Axcan Scandipharma Inc., (Birmingham, AL) and was dissolved in 5% dextrose in water to make a 2.5 mg/ml stock solution. The MMP inhibitor Prinomastat (AG3340) was a gift from Agouron Pharmaceuticals Inc., a Pfizer Company (La Jolla, CA) and was dissolved in acetylated water (pH 2.3) at a final concentration of 3 mg/ml. The solution was sterile filtered, stored at 4°C, and used within 2 weeks. Phorbol 12-myristate 13-acetate was purchased from Sigma (St. Louis, MO) and

Received 1/0/04; revised 2/6/04; accepted 2/19/04.

Grant support: Supported in part by NIH Grants RO-1 CA-31230 (to C. J. Gomer) CA-09897 (to C. J. Gomer), and PO-1 CA-81403 (to H. Shimada and Y. A. DeClerck), the Neil Bogart Memorial Fund of the Martell Foundation for Leukemia, Cancer and AIDS Research (to Y. A. DeClerck and C. J. Gomer), and the Las Madrinas Endowment for Experimental Therapeutics (to C. J. Gomer).

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dissolved in ethanol to make a 10 mg/ml stock solution, 1-10 phenanthroline (OP) was purchased from Sigma and was dissolved in developing buffer, Invitrogen Life Technologies, Inc. (Carlsbad, CA) at a concentration of 1 mM.

Cell Lines and Tumor Model. Mouse mammary carcinoma (BA) cells, mouse brain endothelial cells, mouse macrophages (RAW 264.7), and human fibrosarcoma cells (HT-1080) were grown as monolayers in RPMI 1640 supplemented with 15% FCS and antibiotics. BA tumors were generated by trocar injection of 1-mm³ pieces of tumor to the right flank of 8–12-week-old female C3H/HeJ mice (4).

In Vitro and In Vivo Treatment Protocols. For in vitro treatments, cells were first seeded in Petri dishes and incubated overnight in complete growth medium to allow for cell attachment. Photosensitization experiments were performed, as reported previously (5). Briefly, cells were incubated in the dark with PH (25 µg/ml) for 16 h at 37°C in medium supplemented with 5% FCS. Cells were then incubated for 30 min in fresh growth medium, rinsed in medium without serum, and exposed to broad spectrum (570–650 nm) red light generated by a parallel series of red Mylar-filtered 30-W fluorescent bulbs and delivered at a dose rate of 0.35 mW/cm². Treated cells were re-fed with serum-free medium prior to analysis of MMPs secreted into culture medium. Hypoxia treatments involved incubating cells for 24 h in serum-free medium within a sealed humidified chamber containing 0.6% oxygen at 37°C. Conditioned medium was analyzed for MMPs after treatment. In vivo PDT protocol included an i.v. injection of PH (5 mg/kg), followed 24 h later with tumor irradiation using an argon-pumped dye laser emitting nonthermal red light at 630 nm (4, 5). Light was delivered via a quartz fiber microlens delivery system, and outputs were measured with a power meter. A light dose rate of 75 mW/cm² and total light dose ranging from 0 to 200 J/cm² were used for in vivo PDT tumor treatments. Tumors measuring 6–7 mm in diameter were treated with PDT and monitored three times a week for a detection of tumor recurrence and regrowth. A treatment cure was defined as mice being tumor free for 90 days after PDT. In some experiments, a 100-mg/kg dose of Primocinastat was administered by gastric gavage twice a day for 20 days, either as a single agent or starting immediately after PDT (11).

PDT-Mediated Normal Skin Response. Normal skin response to PDT was evaluated in albinio Swiss Webster mice. A quantitative skin scoring system was used to document photosensitivity by recording the appearance and decline of edema, erythema, and desquamation induced by each treatment (5). The right hind limb of each animal was treated with PDT in the manner identical to that used in the tumor response studies.

Western Immunoblot Analysis. Expression of MMPs was documented by Western immunoblot analysis. BA tumor samples were collected, homogenized with a Polytron in 1 x reporter lysis buffer (Promega, Madison, WI), and evaluated for protein expression, as described previously (4, 5). Protein samples were size-separated on 10% discontinuous polyacrylamide gels and were transferred overnight to nitrocellulose membranes. Filters were blocked for 2 h with 5% nonfat milk and then were incubated for 3 h with mouse monoclonal anti-MMP-1 (Clone 41–I55), MMP-3 (Clone 55–2A4), or MMP-8 (Clone 115–13D2; Oncogene Research Products, Boston, MA); or goat polyclonal anti-MMP-9 (sc-6841); rabbit polyclonal anti-tissue inhibitor of metalloproteinase 1 (anti-TIMP-1; sc-5538), or goat polyclonal anti-extracellular MMP inducer (EMMPRIN; sc-9756; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Filters were then incubated with an antimouse, antirabbit, or antigoat peroxidase-conjugate antibody (Sigma, St. Louis, MO); the resulting complexes were visualized by enhanced chemiluminescence autoradiography (Amersham Life Science, Chicago, IL). Protein loading was evaluated by incubating the same filters with a mouse monoclonal anti-actin antibody (Clone C-4; ICN, Aurora, OH). Autoradiographs were quantified by scanning densitometry.

SDS-PAGE Zymography. The presence of MMP-9 was assayed by gel zymography (12). Aliquots from conditioned medium or tumor lysates were denatured at room temperature in Laemmli Tris-Glycine SDS-PAGE denaturing buffer and were run on 10% gelatin gels (Invitrogen Life Technologies, Inc., Carlsbad, CA). Proteins were renatured by soaking the gels for 30 min in renaturing buffer containing a nonionic detergent (Invitrogen Life Technologies, Inc.). Gels were subsequently incubated overnight with gentle agitation at 37°C in developing buffer which adds back a divalent metal cation required for enzymatic activation of both the proenzyme and active enzyme (Invitrogen Life Technologies, Inc.). Gels were stained with 0.5% Coomassie Blue, destained in a solution containing 10% methanol and 7.5% acetic acid, and dried. Proteins having gelatinolytic activity were visualized as lytic white bands on an otherwise blue gel.

Immunohistochemistry. The localization of MMP-9 expression was examined by immunohistochemistry on 4-μm paraffin-embedded tumor sections pretreated with 3 µg/ml Proteinase K (Roche, Mannheim, Germany) at 37°C for 5 min (11). Slides were incubated overnight at 4°C in the presence of a 1:100 diluted rabbit serum (from rabbits previously immunized for murine MMP-9) and then were incubated with a biotinylated goat antirabbit antibody (Vector Laboratories, Inc., Burlingame, CA) at a dilution of 1:100 for 1 h at room temperature. Slides were incubated with a 1:200 dilution of horseradish peroxidase-conjugated streptavidin (Amersham Biosciences Corp., Piscataway, NJ) for 1 h at room temperature, visualized with 3,3’-diaminobenzidine tetrahydrochloride and counterstained with 1% methylgreen.

Determination of Active MMP-9 in Tumor Tissue. The Biotak MMP-9 activity assay system (Amersham Biosciences Corp.) was used to quantify active MMP-9 in tumor extracts (13). Samples were prepared according to the manufacturer’s instructions. Briefly, tumors were homogenized in 50 mM Tris-HCl buffer (pH 7.4), containing 1 mM monooctylglycerol and were centrifuged at 2000 x g for 10 min. Supernatants were assayed for activated MMP-9 via a modified ELISA assay. Results were normalized to protein concentrations.

Statistics. Statistical analysis of tumor cure rates was based on the log-rank test. Statistical analysis of MMP-9 activity in tumor extracts was performed using a two-tailed Student’s t test. Results with P < 0.05 were considered significant.

Results and Discussion

MMPs are key proteinases involved in the degradation of the extracellular matrix and are identified as playing an essential role in tumor angiogenesis, growth, invasion, and metastasis (7–9). A growing number of studies provide strong evidence of a crucial role for MMP-9 in the process of tumor angiogenesis and growth. MMP-9 is associated with a malignant phenotype in part because of the enzyme’s ability to degrade type IV collagen (14). Recent Kaplan-Meier analysis shows a significant association between MMP-9 expression and shortened cancer-related survival for patients with operable non-small cell lung cancer (15). We initially investigated whether PDT induced the expression and/or activation of MMP-9 because a number of physiological responses associated with PDT, including oxidative stress, inflammatory vascular damage, and hypoxia, elevate levels of this proteolytic enzyme (1, 3). Fig. 1A, panel A, shows MMP-9 expression and activation profiles for tissue lysates collected from one control and three PDT-treated BA mammary tumors. Fig. 1A, panel A, is a Western immunoblot, documenting increased protein expression of MMP-9 in tissue 24 h after PDT. Similar elevations in MMP-1, MMP-3, and MMP-8 were also observed in tumor tissue after PDT (data not shown). We next determined whether the increased protein expression of MMP-9 resulted in a concomitant increase in enzyme activity. MMPs are synthesized as inactivezymogens (pro-MMPs) requiring proteolysis of an NH₂ terminus to be activated (8). Tumor lysates from the control and PDT-treated mice were examined by gelatin zymography to evaluate the presence of active gelatinases. This procedure resolves the gelatinases, MMP-2 and MMP-9 as well as their precursor forms according to their molecular weights and is routinely used for detecting both the latent and active forms of the enzyme (14). Fig. 1A, panel B, shows that PDT-treated tumors had increased gelatinase activity involving both latent MMP-9 (pro-MMP-9) and active MMP-9. Interestingly, basal MMP-2 expression/activity profiles were not changed after PDT. We also used a commercial ELISA kit to obtain quantitative information regarding the extent of MMP-9 activation after PDT. Fig. 1A, panel C, shows that PDT resulted in a 2.5-fold increase in the amount of activated MMP-9 measured in tumor lysates. Zymography profiles for tumor lysates as a function of PDT dose and time after treatment were also obtained. Fig. 1B shows the induction of gelatinase activity with
A 50-J/cm² dose of 630 nm light. Tumor samples were collected 24 h after PDT and were
injected with Photofrin porfimer sodium (PH) and 24 h later, tumors were treated with a
A, PDT light doses (50, 100, 200 J/cm²) were effective at inducing both
electrophoretic mobility pattern than does murine MMP-9. The three
that human MMP-9 has a smaller molecular weight and a faster
pro-MMP-9 and pro-MMP-2 enzymatic bands (11). It should be noted
human HT1080 cells was used to generate positive markers for
the presence of multiple bands in PDT-treated tumor samples
24 or 48 h after treatment. Conditioned medium from untreated
human HT1080 cells was used to generate positive markers for
pro-MMP-9 and pro-MMP-2 enzymatic bands (11). It should be noted
that human MMP-9 has a smaller molecular weight and a faster
electrophoretic mobility pattern than does murine MMP-9. The three
PDT light doses (50, 100, 200 J/cm²) were effective at inducing both
latent and activated forms of MMP-9 as well as inducing increased
gelatinolytic activity at higher molecular weights, possibly because of
protein complexing of metalloproteinases (16). All of the gelatinolytic
bands were completely suppressed by incubating the gels with develop-
ing buffer containing 1-10 phenantroline, which inhibits MMP
activity by forming a complex with Zn (17), thus confirming that the
induced enzymes were metalloproteinases (data not shown). These
results demonstrate for the first time that PDT can increase both the
expression and the enzymatic activity of MMP-9 in BA tumors.

A variety of biomolecules modulate the expression and/or function of MMPs. Prior reports indicate that cellular stress induces expression of
EMMPRIN (18). This cell surface glycoprotein localizes with stromal, endothelial, and tumor cells, and efficiently stimulates the production of MMPs (19). EMMPRIN is a prognostic marker in ovarian carcinoma, and expression correlates with poor prognosis (20). Fig. 2 shows a Western immunoblot demonstrating increased expression of EMMPRIN in tumor tissue 24 h after a 200-J/cm² PDT
dose. These results extend the types of stresses capable of inducing
EMMPRIN expression. A family of TIMPs regulates the enzymatic function of MMPs (12, 21). TIMP-1 is the most ubiquitous of these
endogenous inhibitors and plays an active role in tumor growth,
angiogenesis, invasion, and metastasis by regulating the metabolism of
the extracellular matrix (21). Fig. 2 also shows that PDT blocked
TIMP-1 protein expression in the same tumors in which this treatment
induced increased expression of EMMPRIN. The mechanism(s) by
which PDT induces EMMPRIN expression and/or attenuates TIMP-1
expression are currently unknown. However, PDT induces increased
expression of cyclooxygenase-2 and prostaglandins (1, 5), and, there-
fore, our data, showing that PDT suppresses TIMP-1 expression,
agree with previous studies reporting that TIMP-1 mRNA and protein
levels in endometrial stromal cells and synovial fibroblasts are sig-
nificantly decreased when these cells are incubated with prostaglandin
E₂ (22, 23). The data described above documents that PDT induces
expression of MMPs and EMMPRIN while suppressing TIMP-1
expression in BA tumors. These results indicate there is an imbalance
in the expression of MMPs, EMMPRIN, and TIMP-1 within tumor
tissue after PDT that could decrease treatment effectiveness and
contribute to tumor recurrences.

We next used immunohistochemistry to identify the site(s) of
MMP-9 expression in tumor tissue after PDT. MMPs can be synthe-
sized by stromal cells and infiltrating inflammatory cells within tu-
nors (8, 24). Stromal-derived MMP-9 contributes to angiogenesis in
neuroblastoma that is orthotopically transplanted into severe com-
bined immunodeficient mice; it does this by promoting blood vessel
morphogenesis and pericyte recruitment (11). Photofrin-mediated
PDT of solid tumors induces host cell infiltration associated with an
intense inflammatory response involving the expression of interleukin
1β, tumor necrosis factor α, and interleukin 6 (3). Fig. 3A, panel A,
shows sparse MMP-9 staining localized to stromal elements or rare inflammatory cells in control, untreated BA tumors. PDT caused extensive infiltration of MMP-9-expressing inflammatory cells within the treated tumor when examined 6 h after treatment (Fig. 3A, panel B) as well as expression of MMP-9 in tumor vascular endothelial cells when examined 24 h after treatment (Fig. 3A, panel C). We used gelatin zymography to examine whether MMP-9 expression patterns in PDT-treated cells were similar to what we observed after in vivo PDT of tumors. Fig. 3B shows that, under control conditions, cultured BA tumor cells secreted only detectable levels of MMP-2, whereas RAW 264.7 macrophages and mouse brain capillary endothelial cells secreted both MMP-9 and MMP-2. We observed that endothelial cells and macrophages were effective sources of both gelatinases when challenged with the positive control phorbol 12-myristate 13-acetate (PMA). An increase in the expression of pro-MMP-9 and pro-MMP-2 as well as in activated MMP-9 was detected in the culture medium from PDT-treated endothelial cells. We also observed a decrease in MMP expression in PDT-treated macrophages that was probably due to the acute sensitivity of these cells to PDT. This suggests that the increased expression of MMP-9 observed in tumor tissue after PDT involves the influx of MMP-9-expressing inflammatory host cells, as opposed to direct PDT-induced expression of MMP-9 in inflammatory cells present within tumor tissue at the time of treatment. These results demonstrate that endothelial cells and infiltrating host cells are sources of MMP-9 in PDT-treated BA tumors.

Tumor growth requires modification of the extracellular matrix, as well as of enhanced angiogenesis; and these processes are positively correlated with MMP expression (24). These observations have fueled the evaluation of endogenous and synthetic inhibitors of MMPs in the treatment of cancer. Unfortunately, Phase III clinical trials using MMP inhibitors have been disappointing (24). Most studies used patients with advanced disease whereas preclinical studies indicate that inhibitors have minimal effectiveness in treating large lesions (7). Data suggest that the most efficacious use of MMP inhibitors is during early neoplastic progression and particularly in combination with cytotoxic agents that can efficiently reduce tumor mass (8). The use of PDT, which rapidly decreases the fraction of viable tumor tissues, appears particularly suited to adjunctive therapy involving MMP inhibitors. We examined the therapeutic effectiveness of combining PDT with Prinomastat (AG-3340), a potent synthetic MMP inhibitor with significant affinities for MMP-2 and MMP-9 (11, 24, 25). Preclinical studies show that Prinomastat produces growth delays in a variety of tumors including neuroblastoma, as well as in cancers of the lung, breast, colon, brain, and prostate (11, 25). Prinomastat inhibits tumor angiogenesis and cell proliferation and enhances the efficacy of carboplatin and Taxol (25). In the present experiments, mice with transplanted BA tumors measuring 5–7 mm in diameter were treated with PH-mediated PDT at the light dose of 200 Joules/cm². This PDT dose routinely results in ~20% long-term cures and was used so that potential changes in tumor response after adjunctive therapy could be readily measured. Immediately after PDT, one group of mice were started on a 20-day regimen of Prinomastat delivered twice a day at a dose of 100 mg/kg. Tumor response was monitored for a 90-day evaluation period. Fig. 4A shows the percentage of tumor-free mice as a function of days after treatment for mice receiving PDT alone or PDT plus Prinomastat. PDT alone resulted in a 20% cure rate whereas the combination of PDT and Prinomastat resulted in 46% long-term cures. This difference was found to be statistically significant.
Prinomastat. The 50-J/cm² PDT light dose resulted in only modest skin photosensitivity that again was not modified by adjunctive use of Prinomastat. MMPs are inducible in various cells found in skin including fibroblasts, keratinocytes, macrophages, and endothelial cells. However, we did not observe any difference in the magnitude or repair of skin photosensitivity for treatments using PDT alone or PDT plus Prinomastat, which suggests that MMP expression does not modulate PDT-mediated normal skin photosensitivity. In conclusion, our results demonstrate that a combination-therapy approach using PDT and an MMP inhibitor can increase the therapeutic efficacy.

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